Genetic Engineering for Productivity in the Fermentation of Xylose to Ethanol

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INTRODUCTION

With the increased awareness of the limitations in the world supplies of petroleum came an increased interest in gasoline extenders and additives. The use of ethanol as an extender on a national scale is associated with advantages (1,2): the security of fuel sources; a more favorable foreign trade balance; and the renewability of the raw materials for production. Although ethanol can be produced either from a petroleum component or by fermentation, the latter source is the only one associated with all of the advantages above. The most attractive raw materials for ethanol production in this country are corn sugar, and sugar derived from wood and crop residues (lignocellulosic materials). Corn ($98-$104 per ton), (2) can be competitive only in special circumstances, such as government subsidy. However, lignocellulosic material ($20-$70 per ton) could be competitive, if the technical limitations associated with processing and fermentation could be overcome. Although the approximate price of corn-derived ethanol is currently $1.20 per gallon, it is estimated that the cost of fuel ethanol from lignocellulosic materials should be $0.60-$0.80 per gallon (3).

The most productive and cost effective processing of lignocellulosic material currently consists of mild acid hydrolysis to release a fraction containing primarily xylose (15%-40% of dry weight) followed by strong acid treatment to release the glucose (30%-60%), (4,5,6). The sugars are then fermented, usually separately, to ethanol. However, there are two steps in particular that limit the productivity and increase the cost of this process: the hydrolysis of cellulose and the fermentation of xylose. The most promising solution to the former problem is enzymatic hydrolysis, but currently the enzymes remain too expensive and the hydrolysis is incomplete. The problem with xylose fermentation results from a poor overall yield. It is estimated that the efficient conversion of xylose would reduce the cost of ethanol by about $0.40/gal (7).

A number of both yeasts and bacteria will convert xylose to ethanol and other products (8,9,10), but most of the attention has been given to three yeast strains: *Pachysolen tannophilus, Candida shehatae,* and *Pichia stipitis*. Yeast has the advantages of growing in a simple, inexpensive medium; growing at a low pH to minimize contamination; and being the subject of much past experience. Although both bacteria and yeast generally convert D-xylulose to hexose (as the phosphate esters) and convert the hexose to ethanol by the classic fermentation pathway, they differ with respect to the methods used to convert D-xylose to D-xylulose. Most bacteria carry out the conversion with a single enzyme, xylose isomerase; whereas most yeast reduce the xylose to xylitol with xylose reductase and subsequently reoxidize it to xylulose with xylitol dehydrogenase.

The principal limitation to the ethanol yield in the yeast fermentation is the accumulation of xylitol in molar amounts equal to

or exceeding that of ethanol (11). Furthermore, the accumulated xylitol is not further utilized. Fermentation under micro-aerobic conditions reduces the amount of xylitol (12,13) accumulated, but the oxygen also promotes the further oxidation of ethanol.

The ethanol yield has been improved by the addition to the medium of fatty acids (14), polyethylene glycol (15), azide (16), or acetone (17). In addition mutants with increased yield have been reported (18,19,20). However, none of these strategies seems to have become widely used in practice. Xylose isomerase has been utilized in the fermentation to facilitate the conversion of xylose to xylulose (21), but neither the equilibrium of the reaction nor the pH optimum of the enzyme is favorable (22). Several workers have described strains of *Saccharomyces cerevisiae* (23,24) and *Schizosaccharomyces pombe* (25) into which the xylose isomerase gene has been cloned, but the productivity was low presumably because of low levels of transport systems, xylulokinase, transketolase, and transaldolase.

The most important unanswered questions deal with the causes of xylitol accumulation and its non-utilization. Several investigators have attributed xylitol accumulation to the fact that the majority of the xylose reductase activity requires NADPH, whereas the xylitol dehydrogenase requires NAD (26,27). However, if this nucleotide imbalance were the only cause, the utilization of xylose would quickly stop, because of the low concentration of intracellular nucleotides.

The poor xylitol utilization is inconsistent with its status as an intermediate. Although the utility of oxygen and acetone in xylitol conversion, as well as its redox status, indicates that an electron acceptor is necessary, xylitol still accumulates to a very significant extent and the utilization of extracellular xylitol remains very poor in the presence of oxygen.

The present work was undertaken to provide answers to these questions and to utilize the answers to design and implement a strategy of metabolic and genetic engineering to develop a strain of *Pachysolen tannophilus* to ferment xylose efficiently.

RESULTS

Whole, resting cells of *Pachysolen tannophilus* converted xylose to ethanol (yield, 1.11 mole/mole) but did not convert xylitol (28). However, a cell-free extract, prepared by French pressure cell and low-speed centrifugation, produced ethanol from both xylose (yield, 1.1 mole/mole) and xylitol (yield, 1.64 mole/mole; theor, 1.67 mole/mole). These results supported the hypothesis that the cell permeability to xylitol limits its utilization and the fact that nystatin, amphotericin B, and filipin; all agents that increase the cell-membrane permeability of yeast and fungi (29,30), increased the rate 40-fold.

In order to simplify the investigation of the requirements for xylose metabolism the putative pathway was separated into two parts by the inclusion of xylose isomerase (Spezyme GI, Finnsugar Biochemicals, Inc.) in the incubations with the fractions of the cell-free extract. Preliminary experiments showed that ethanol formation from xylose by the crude cell extract was stimulated 9-fold by added xylose isomerase.

In the Presence of Xylose Isomerase the activity of the HMW fraction, prepared by gel filtration of a soluble fraction of the cell-free extract, was restored by the addition of both ADP and NAD^+ (Table 1). Although NAD^+ stimulated ethanol formation in this preparation (fraction A), its complete removal required treatment three times with

gel filtration and once with charcoal (fraction B, Table 1).

In contrast to our previous results with xylitol (28) xylose is metabolized in the absence of oxygen, and xylose is metabolized by soluble fractions of the cell-free extract from which the membranes and organelles have been removed by centrifugation.

Although the addition of NAD^+ and ADP restored the production of ethanol from xylose by the HMW fraction, in the presence of xylose isomerase, their addition failed to restore ethanol formation from xylose by the same fraction in the absence of xylose isomerase. In addition, the inclusion of $NADP^+$, NADPH, or ATP in addition to NAD^+ and ADP, as well as the inclusion of a variety of other coenzymes and metal ions in the presence of NAD^+, $NADP^+$ and ADP failed to restore activity.

Because of the fact that added NADPH was rapidly consumed by the HMW fraction in the absence of xylose and the fact that, at higher concentration (>100 μM), both NADPH and $NADP^+$ inhibited ethanol formation from xylose by the unfractionated cell extract, three NADPH generation systems were tested to maintain a constant supply of the reduced coenzyme at low concentration: partially purified transhydrogenase from *E. coli K-12*, NADP-linked isocitrate dehydrogenase from porcine heart, and NADP-linked alcohol dehydrogenase from *Thermoanaerobium brockii*. Although transhydrogenase activity ineffective in the restoration of ethanol production, both the NADP-linked isocitrate dehydrogenase and the alcohol dehydrogenase stimulated the formation of ethanol from xylose by about 60 and 80 fold respectively (Table 2).

A number of factors contribute to the causes of xylitol accumulation. Certainly the fact that xylose reductase requires NADP whereas xylitol dehydrogenase requires NAD (nucleotide imbalance) is one of the factors. Another is certainly the fact that extracellular xylitol is not normally utilized by whole cells.

In experiments containing purified cell-free fraction, xylose isomerase, NAD and ADP, xylitol was produced during xylose metabolism (Table 3). Furthermore, the gas chromatographic assay for ethanol contained an additional peak that had the same retention time as acetaldehyde. Therefore, some of the NADH generated by glyceraldehyde phosphate dehydrogenase is reoxidized in the generation of xylitol, presumably from xylulose by xylitol dehydrogenase. The addition of $NADP^+$ had little effect on the amount of ethanol made from xylose, but it increased the xylitol accumulation by 2.5-fold; increased the xylose consumption by 2-fold; and eliminated the acetaldehyde peak (Table 3). Therefore, most of the xylitol is generated by xylose reductase in these extracts containing all of the cofactors and presumably in the whole cells.

Xylose is reduced to xylitol with electrons from the pentose-hexose cycle, but the poor coupling of the rate with the rate of xylitol dehydrogenase causes xylitol to accumulate faster than it is utilized. The intracellular xylitol leaks out of the cell and becomes diluted in the medium, from which it is not reutilized because the transport back into the cell is limiting. The intracellular xylitol can be metabolized, if oxygen is present. Thus oxygen is required for optimum xylose metabolism.

The logical strategy for process improvement according to the results above would be the transformation and expression of the gene for xylose isomerase in one of the xylose-fermenting yeasts. However, it would also be necessary to inhibit both the xylose reductase and xylitol dehydrogenase, in order to prevent xylitol accumulation. However, the transformants should be able to ferment xylose to ethanol

anaerobically.

The genetic engineering strategy to accomplish these objectives takes advantage of homologous recombination in yeast, at least in *Saccharomyces cerevisiae*, in which genetic material with homologous DNA sequences will insert in the homologous gene with high frequency. Therefore, the desired genetic construct will contain the gene of interest, xylose isomerase plus a suitable promoter, flanked on either end with DNA sequences homologous to those for xylose reductase or those for xylitol dehydrogenase. These constructs should take advantage of the high frequency of recombination to increase the transformation frequency as well as inactivate the target gene.

In preliminary experiments a gene conferring antibiotic resistance, e.g. kanamycin, will be used in place of the xylose isomerase gene because the feasibility of the strategy and the optimal experimental conditions can be determined with an easily selectable marker. The successfully transformed strain should be resistant to the antibiotic and be unable to grow on xylose. In subsequent experiments the gene for antibiotic resistance will be put in tandem with the gene for xylose isomerase. In the latter case the successfully transformed strain should be antibiotic resistant and grow anaerobically on xylose.

The identification of and the determination of the sequences for xylose reductase and xylitol dehydrogenase in *Pachysolen tannophilus* require a probe with an homologous DNA sequence. The gene for each of the two enzymes from *Pichia stipitis* has been identified and sequenced (31,32), and part of the amino acid sequence of xylose reductase from *Pachysolen tannophilus* is known (33). A homologous probe for the *Pachysolen* gene for xylose reductase has been designed and constructed by back translating the amino acid sequence of a region that is highly homologous with the *Pichia* sequence according to the most frequent codon usage of *Pachysolen*. This probe was made radioactive and used to test binding to whole *Pachysolen* DNA, a restriction enzyme digest of *Pachysolen* DNA, and to the DNA from clones from a xylose-induced cDNA library of *Pachysolen* DNA. The positive results from each experiment are being tested further with this and similar probes, and the appropriate DNA will be sequenced to identify the limits of the coding sequences and the promoter for the gene.

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Table 1. Effects of Sephadex column and charcoal treatment of the high speed supernatant fraction (HSSF)[a]

Fraction	Additional Components		EtOH mg/ml
HMW	-	-	<0.01
HMW	-	NAD^+	<0.01
Fraction A[b]	ADP	-	0.12
Fraction A	ADP	NAD^+	0.64
Fraction B[c]	ADP	-	<0.01
Fraction B	ADP	NAD^+	0.62

[b]Fraction A, the HMW fraction of the high speed supernatant fraction has undergone two consecutive gel filtration treatments.
[c]Fraction B, Fraction A was treated with charcoal.

Table 2. Restoration of ethanol formation from xylose in the HMW fraction of the crude cell extract in the presence of an NADPH generation system[a]

Reaction Mixture	Substrate	EtOH Made mg/ml
None	Xylose	<0.01
IC[a] + ICDH[a]	none	0.057
IC + ICDH	Xylose	0.710
cPen-OH[a] + ADH[a]	none	0.016
cPen-OH + ADH	Xylose	0.925
TH[b] + NADPH (0.1 mM)	Xylose	<0.01

[a]IC, sodium isocitrate; ICDH, $NADP^+$-linked isocitrate dehydrogenase; cPen-OH, cyclopentanol; ADH, $NADP^+$-linked alcohol dehydrogenase; TH, transhydrogenase preparation.

Table 3. Comparison of the ratio of formation of ethanol to xylitol by different systems[a]

	Xylose Used μmole	Xylitol Made μmole	EtOH Made μmole	Ratio
Whole Cell				5.75[a]
Crude Extract				3.63[a]
HSSF				0.63[a]
Fraction B[b] + XI[b] + ADP + NAD^+	15.4	7.9	11.0	1.39
Fraction B + XI + ADP + NAD^+ + $NADP^+$	29.1	19.9	9.7	0.49

[a]Xu and Taylor (1993a).
[b]Same as in Table 1; XI, xylose isomerase.